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Evaluation of the microscopic observation drug susceptibility assay for detection of *Mycobacterium tuberculosis* resistance to pyrazinamide

L. D. Ghiraldi[1], P. A. Z. Campanerut[2], F. L. E. Sposito[2], D. N. Sato[3], C. Q. F. Leite[4], M. Hirata[5], R. D. C. Hirata[5] and R. F. Cardoso[6]

1) Postgraduate Programme in Health Science, 2) Postgraduate Programme in Biosciences Applied to Pharmacy, State University of Maringá, Paraná, 3) Adolfo Lutz Institute, Ribeirão Preto, 4) School of Phamaceutical Science, Paulista State University, Araraquara, 5) School of Pharmaceutical Science, University of São Paulo, São Paulo and 6) Department of Clinical Analyses and Biomedicine, State University of Maringá, Paraná, Brazil

Abstract

The microscopic observation drug susceptibility assay (MODS) was evaluated to determine susceptibility to pyrazinamide in *Mycobacterium tuberculosis*, and compared with the broth microdilution method (BMM), absolute concentration method (ACM), and pyrazinamidase (PZase) determination. We tested 34 *M. tuberculosis* clinical isolates (24 sensitive and eight resistant to pyrazinamide) and the control strains *M. tuberculosis* H37Rv (ATCC 27294) and *Mycobacterium bovis* AN5. The MODS, BMM, ACM and PZase determination provided results in average times of 6, 18, 28 and 7 days, respectively. All methods showed excellent sensitivity and specificity ($p < 0.05$). Of the methods studied, the MODS proved to be faster, efficient, inexpensive, and easy to perform. However, additional studies evaluating the MODS in differentiating pyrazinamide-resistant and pyrazinamide-susceptible *M. tuberculosis* must be conducted with a larger number of clinical isolates.

Keywords: Microscopic observation drug susceptibility assay (MODS), pyrazinamide, resistance, susceptibility testing, tuberculosis
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Corresponding author: R. F. Cardoso, Departamento de Análises Clínicas e Biomedicina, Universidade Estadual de Maringá, Avenida Colombo, 5790, 87020-900 Maringá, Paraná, Brazil
E-mail: rfcardoso@uem.br

Introduction

Tuberculosis (TB) is a worldwide health problem. It is estimated that 9.27 million cases occurred in 2007, and approximately 15% of these involved co-infection with human immunodeficiency virus [1]. Pyrazinamide is an important drug that is used in combination with isoniazid, rifampicin and ethambutol in the first-line treatment of TB [2]. Pyrazinamide needs to be converted to pyrazinoic acid by pyrazinamidase (PZase), which is effective against a population of semidormant *Mycobacterium tuberculosis* in the acid environment inside macrophages that cannot be reached *in vivo* by other drugs [3].

Pyrazinamide susceptibility testing *in vitro* is somewhat difficult to perform, because the acid pH of the medium required to maintain drug activity inhibits bacterial growth [4]. Conventional pyrazinamide susceptibility testing by the Lowenstein–Jensen (L-J) proportion method is most often used for *M. tuberculosis*, but this technique requires a minimum of 3–4 weeks to produce results [5,6]. Furthermore, we need to consider the fact that when the drug is included in the L-J medium and exposed to high temperature (85°C) during the preparation of the medium, its potency may be reduced [7].

New methods have been proposed recently for *M. tuberculosis* susceptibility testing, including the resazurin microtitre assay plate [8], the microplate Alamar Blue assay [9], the 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide assay [10], the broth microdilution method (BMM) [11], and the microscopic observation drug susceptibility assay (MODS) [12,13]. These methods have the advantage of using a liquid medium, which can provide results in a shorter time.

The MODS [12] is fast and easy to perform. It also has a low cost and allows simultaneous detection of the bacilli and

the incorporation of drugs for the susceptibility testing. The method is based on the principle that *M. tuberculosis* grows faster in a liquid medium, and its characteristic growth 'cording formation' can be observed through an inverted light microscope [12–15]. Several studies have used the MODS to determine susceptibility to isoniazid and rifampicin [12,13,15–17], and it seems to be reliable as compared with the reference method. However, it has not been applied to detect susceptibility to pyrazinamide.

The aim of our study was to evaluate the application of the MODS for the determination of susceptibility to pyrazinamide in *M. tuberculosis*, and to compare it with the BMM, the absolute concentration method (ACM), and determination of PZase activity.

Materials and Methods

Mycobacterial strains

The pyrazinamide-susceptible strain *M. tuberculosis* H37Rv (ATCC 27294) and the pyrazinamide-resistant strain *Mycobacterium bovis* AN5 were used as controls in the susceptibility test for pyrazinamide. *Mycobacterium avium* (ATCC 13950) and *M. bovis* AN5 were used as positive and negative controls, respectively, for determination of PZase activity.

A total of 32 *M. tuberculosis* clinical isolates were selected from the mycobacteria collection of the Clinical Bacteriology Laboratory in the Clinical Analysis and Biomedicine Department of Maringá State University. Of these isolates, 24 were susceptible and eight were resistant to pyrazinamide, as assessed by the proportion method in L-J medium (pH 5.5) with a pyrazinamide concentration of 100 mg/L as a cut-off for resistance [18]. Automated DNA sequencing of a 1200-bp segment including the entire *pncA* open reading frame, as well as its regulatory region, was previously carried out [19] in pyrazinamide-resistant *M. tuberculosis* isolates.

Pyrazinamide solution

A stock solution of 20 000 mg/L pyrazinamide (Sigma, St Louis, MO, USA) in distilled water was prepared, filter-sterilized, and stored at −20°C until use. At the time of use, the stock solution was diluted to give the MICs for the MODS, BMM, and ACM.

MODS

The MODS was performed as previously described [16] in 24-well sterile plates (TPP, Trasadingen, Switzerland), in triplicate. Middlebrook 7H9 medium (Difco Laboratories, Detroit, MI, USA) supplemented with OADC Enrichment (BBL/Becton-Dickinson, Sparks, MD, USA) was prepared as previously described [12], and the pH was adjusted to 6.0 [19]. One millilitre of drug-free medium was added to each well, and the pyrazinamide stock solution was diluted to obtain final concentrations of 6.25–3200 mg/L. One hundred microlitres of mycobacterial inoculum, previously standardized according to 1 McFarland turbidity and diluted to 10^{-3} [16], was inoculated into each well of drug-containing medium, and also into the wells of the mycobacterial control. Each plate was covered with its lid and sealed along its edge with polyethylene tape. The plates were placed in a plastic bag to prevent evaporation, and incubated at 35°C in normal atmosphere. Mycobacterial growth was observed daily with an inverted light microscope at ×40 magnification (Olympus, CK 40) from the 3rd to the 15th day of incubation [13,16]. Mycobacterial growth was defined as the emergence of visually characteristic serpentine growth. The MIC results were interpreted on the day when distinct growth could be observed in the control wells for each *M. tuberculosis* isolate. The isolates that showed MICs ≤100 mg/L were considered to be susceptible to pyrazinamide [19].

BMM

The BMM was performed as previously described [11], in triplicate. First, 200 μL of distilled water was added to the outer wells of the 96-well microplates (Kartell, Milan, Italy). One hundred microlitres of Middlebrook 7H9 medium (Difco Laboratories), supplemented with OADC Enrichment (BBL/Becton-Dickinson) and with the pH adjusted to 6.0 [19], was added to each well. The pyrazinamide stock solution was diluted to obtain concentrations from 12.5 to 3200 mg/L. Five microlitres of bacterial inoculum standardized to 0.5 McFarland turbidity and further diluted to 10^{-2} [11] was inoculated into the wells of drug-containing media and also into the control wells. Each plate was covered with its lid and sealed along its edge with polyethylene tape. The plates were placed in a plastic bag to prevent evaporation, and incubated at 35°C in a normal atmosphere. The readings were made after 14, 20 and 28 days of incubation through visual observation of the growth. The MIC was defined as the lowest pyrazinamide concentration that exhibited no growth by visual reading, and the isolates were considered to be susceptible to pyrazinamide if their MICs were ≤100 mg/L [19].

ACM

The ACM was performed in triplicate in L-J medium (Difco Laboratories). The L-J medium was prepared according to the manufacturer's instructions, and the pH was adjusted to 5.2 with sterile 5 M hydrochloric acid (Quimex, São Paulo, Brazil) [5]. The pyrazinamide stock solution was diluted to

obtain concentrations from 6.25 to 3200 mg/L, and the medium was subjected to coagulation for 45 min at 85°C. One hundred microlitres of standardized bacterial inoculum with Mcfarland turbidity 1 and further diluted to 10^{-3} in saline was inoculated onto L-J slant medium containing different pyrazinamide concentrations. A mycobacterial growth control for each *M. tuberculosis* isolate was carried out in L-J slant medium, at pH 5.2 and 6.8 without pyrazinamide [19]. All media were incubated at 35°C for 28 days and inspected weekly. The MIC was expressed in terms of the lowest concentration of the drug that inhibited mycobacterial growth. Isolates showing MICs ≤100 mg/L were considered to be susceptible [19].

Determination of PZase activity

PZase activity was assayed qualitatively as previously described [5], with Dubos broth medium (Difco Laboratories) containing 100 mg/L pyrazinamide. The test was performed in duplicate for each *M. tuberculosis* isolate, and incubated at 35°C for 7 and 14 days. The tests were read by addition of 1 mL of freshly prepared 1% ferrous ammonium sulphate solution (Quimibrás, Rio de Janeiro, Brazil) and maintained at room temperature for 1 h. The test result was considered to be positive for PZase activity if a pink band appeared in the agar medium.

Statistical analysis

The software EpiInfo (Centers for Disease Control and Prevention, Atlanta, GA, USA), version 3.32, was employed to analyse the data, at a significance level of 5%. The associations of the MODS and BMM and PZase activity were assessed by Fisher's exact test.

Results

The MIC, PZase activity and *pncA* mutation results for the clinical *M. tuberculosis* isolates resistant to pyrazinamide are listed in Table 1. The pyrazinamide-resistant *M. tuberculosis* isolates showed MICs ≥3200 mg/L and ≥800 mg/L by the MODS and the ACM, respectively. The BMM detected six (75%) resistant isolates, with MICs ≥800 mg/L. Two resistant isolates did not show growth by this method.

Twenty-one (87.5%) of 24 pyrazinamide-susceptible *M. tuberculosis* clinical isolates showed growth by the MODS, 22 (91.6%) by the BMM, and 24 (100%) by the ACM. All of the susceptible isolates showed MICs ≤100 mg/L (Table 2).

The PZase test gave positive results for all pyrazinamide-susceptible isolates and for one pyrazinamide-resistant *M. tuberculosis* isolate (Tables 1 and 2).

TABLE 1. MICs for pyrazinamide, pyrazinamidase (PZase) activity and *pncA* mutations in pyrazinamide-resistant clinical isolates of *Mycobacterium tuberculosis* and control strains (*M. tuberculosis* H37Rv and *Mycobacterium bovis* AN5)

	Pyrazinamide MIC (mg/L)				*pncA* mutations	
Isolates	MODS	BMM	ACM	PZase	N	AA
H37Rv	100	≤12.5	100	+	NM	NM
AN5	>3200	>3200	>3200	–	G169C	H57N
3614	3200	1600	1600	–	T464G	V155E
69A	>3200	>3200	>3200	–	A287C	K96T
15109	>3200	800	1600	–	T490C	S164P
1137	>3200	NG	800	–	T359G	L120R
73A	>3200	800	1600	–	T175C	S59P
3224	>3200	NG	800	+	NM	NM
71A	>3200	800	1600	–	A11G	NA
5505	>3200	>3200	>3200	–	Deletion	

AA, amino acid changes in pyrazinamidase; ACM, absolute concentration method; BMM, broth microdilution method; MODS, microscopic observation drug susceptibility assay; N, nucleotide changes in the *pncA* gene; NA, not applicable; NG, no growth; NM, no mutation.

TABLE 2. MICs for pyrazinamide and pyrazinamidase (PZase) activity in pyrazinamide-susceptible clinical isolates of *Mycobacterium tuberculosis* and control strains (*M. tuberculosis* H37Rv and *Mycobacterium bovis* AN5)

	Pyrazinamide MIC (mg/L)			
Isolates	MODS	BMM	ACM	PZase
H37Rv	100	≤12.5	100	+
AN5	>3200	>3200	>3200	–
1264	100	100	100	+
1118	100	100	12.5	+
23657	6.25	25	100	+
1s	50	12.5	100	+
3s	100	12.5	100	+
4s	100	50	100	+
5s	100	12.5	100	+
7s	12.5	12.5	100	+
9s	6.25	12.5	25	+
10s	6.25	12.5	100	+
13s	12.5	12.5	100	+
14s	12.5	12.5	100	+
16s	25	12.5	100	+
TB 19	100	12.5	100	+
TB 27	NG	12.5	100	+
TB 40	100	50	50	+
TB 43	NG	12.5	100	+
TB 46	12.5	25	100	+
TB 49	12.5	12.5	100	+
TB 54	6.25	12.5	100	+
TB 57	6.25	12.5	100	+
TB 71	50	12.5	100	+
TB 80	NG	12.5	100	+
Mgá01	12.5	12.5	100	+

ACM, absolute concentration method; BMM, broth microdilution method; MODS, microscopic observation drug susceptibility assay; NG, no growth.

MIC results were available within a mean of 6.6 days (range, 4–15 days) for the MODS, 18 days (range, 14–28 days) for the BMM, and 28 days for the ACM. The MIC results were obtained in 7 days for 82.8% of the isolates tested by the MODS, and in 14 days for 62.8% of the isolates tested by the BMM (Table 3).

TABLE 3. Performance and detection time of the microscopic observation drug susceptibility assay (MODS), the broth microdilution method (BMM), and pyrazinamidase (PZAse) activity as compared with the absolute concentration method

Method	Relative sensitivity, % (95% CI), *n*	Relative specificity, % (95% CI), *n*	Agreement, % (95% CI), *n*	p-Value[a]	Time (days)
MODS	100 (68.77–100.00), 29	100 (86.71–100.00), 29	100 (90.19–100.00), 29	0.000000233	6.6
BMM	100 (60.70–100.00), 30	100 (88.27–100.00), 30	100 (90.50–100.00), 30	0.000001684	18
PZase	87.50 (51.97–99.37), 32	100 (88.27–100.00), 32	96.88 (85.54–99.84), 32	0.000002377	7

[a]Mid-p exact test.

The performance of the MODS, the BMM and PZase determination for detection of resistance to pyrazinamide was determined when selecting the ACM as standard. The MODS and the BMM sensitivity and specificity were complete (100%). For PZase activity, they were 87.50% and 100%. There was no significant difference among the tests ($p < 0.05$) (Table 3).

Discussion

Although pyrazinamide is a first line anti-TB drug used in the first 2 months of TB treatment, the susceptibility test for pyrazinamide in *M. tuberculosis* poses some difficulties, because it has low reproducibility, owing to the drug being active only in a relatively low-pH medium [4]. This low-pH environment may decrease the colony count by nearly 50% for *M. tuberculosis* as compared with a neutral pH environment, in the proportion method [18]. Pyrazinamide is very important in TB treatment, because it has a sterilizing effect by killing *M. tuberculosis* in the acid environment during active inflammation. In this sense, it is very important to have a fast, sensitive and easy susceptibility test for pyrazinamide, to be carried out not only before treatment, but mainly in the course of the treatment for the management of multidrug-resistant TB [20].

In the present study, the introduction of the MODS (pH 6.0) for the detection of susceptibility to pyrazinamide in *M. tuberculosis* showed 100% agreement with the ACM. Although the number of pyrazinamide-resistant isolates evaluated was small ($n = 8$), all of them showed MICs ≥800 mg/L by the MODS and the ACM. Mengatto *et al.* [16], comparing the MODS with the proportion method to detect susceptibility to isoniazid at concentrations of 0.1 and 0.4 mg/L, obtained 100% and 88% agreement, respectively. For rifampicin, these authors obtained 100% agreement for the concentrations of 1 and 0.5 mg/L.

An important factor in detection of the susceptibility of *M. tuberculosis* to the drugs available to treat TB is the time needed to read the test. The MODS showed a mean time of 6.6 days for the detection of pyrazinamide susceptibility, and was faster than the BMM and the ACM, which required, on average, 18 and 28 days, respectively.

One of the advantages of using the MODS to detect pyrazinamide susceptibility is the ease of reading the test. The growth of *M. tuberculosis* is detected by observation of the characteristic growth in a cording formation, which requires only an inverted light microscope [12]. It is not necessary to use sophisticated equipment or reagents, making it easier to use the susceptibility test in laboratories with limited financial resources, particularly in developing countries, where the rates of multidrug-resistant TB and extremely drug-resistant TB are increasing [1]. It is important to emphasize that the same safety precautions as used to perform cultures in liquid medium are necessary for reading the MODS, and the plates must be sealed when the test is read, in order to decrease the risk of aerosol formation.

In our study, we observed that MICs in the BMM [11] were generally lower than those obtained by the ACM. One pyrazinamide-susceptible and two pyrazinamide-resistant isolates showed no growth by this method. Both situations, the non-mycobacterial growth and the lower MIC values, can be explained by the difficulty in obtaining mycobacterial growth in Middlebrook 7H9 at pH 6.0, and also the subjectivity in the reading test, which is based on visual observation of growth. Another disadvantage of the BMM for pyrazinamide susceptibility detection is the time needed to read the test, which averaged 18 days.

Mengatto *et al.* [16] obtained good results with the BMM for the detection of susceptibility to isoniazid and rifampicin, with a sensitivity higher than 90% as compared with the proportion method in L-J medium. On the other hand, the results for streptomycin, and especially for ethambutol, showed lower agreement. In general, depending on the method used, the MIC values were not equal, although they showed little difference, but this does not change the susceptibility categorization. This finding may be explained by differences in the mycobacterial inoculum, medium, and time of reading, and whether visual or microscopy observation was used.

The determination of PZase activity is intimately related to resistance to pyrazinamide. The lack of this enzyme in the bacillus prevents pyrazinamide from being converted into pyrazinoic acid and then conferring resistance on the *M. tuberculosis* complex [21]. Mutations in *pncA*, coding for PZase, may provide resistance to pyrazinamide, owing to non-conversion of the pyrazinamide to its active metabolite by PZase [22,23]. All of the pyrazinamide-susceptible *M. tuberculosis* isolates tested in our study were positive for PZase activity. Of the eight clinical isolates characterized as resistant to pyrazinamide, seven (87.5%) had negative results for PZase activity, and had *pncA* pontual mutations or even total deletion of the gene. Other studies [19,22–24] have also found a relationship between the presence of a mutation in *pncA* and negative results for PZase activity. Only one pyrazinamide-resistant isolate was positive for PZase activity, despite showing an MIC value characterized as resistant by the MODS (>3200 mg/L) and the ACM (>800 mg/L). The same isolate did not grow in the BMM. This pyrazinamide-resistant isolate did not have mutations in *pncA* and its regulatory region [19], which leads us to consider the possibility of the existence of other mechanisms of resistance to pyrazinamide, such as changes in pyrazinamide uptake or increased efflux of the active drug [19,24–27].

The sensitivity of PZase activity determination found in our study (87.5%) is similar to levels reported previously [21,28], indicating that this is an efficient method for the detection of pyrazinamide resistance in *M. tuberculosis*. The great utility of PZase activity determination for the detection of resistance to pyrazinamide resides in its high specificity, rapidity, and ease of performance [21].

Our results show that, of the methods used to detect susceptibility to pyrazinamide in *M. tuberculosis*, the MODS proved to be faster, efficient, inexpensive, and easy to perform. Additional studies with larger numbers of isolates resistant to pyrazinamide and with MIC values lower than those used in this study will enable a better understanding of the utility of the MODS in differentiating pyrazinamide-resistant and pyrazinamide-susceptible *M. tuberculosis*. Such studies will enable the application of this method for the rapid detection of resistance of *M. tuberculosis* to pyrazinamide in clinical laboratories with limited financial resources.

Transparency Declaration

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